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Customer No. 22,852
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Evy LUNDGREN-AKERLUND	)	Group Art Unit: 1644
	)	
Application No.: 10/517,210	)	Examiner: Maher M. Haddad
	)	
Filed: March 9, 2005	)	
	)	
For: MARKER FOR STEM CELLS AND ITS USE	)	Confirmation No.: 4342
	)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. § 1.132

I, Evy Lundgren-Akerlund, do hereby make the following declaration:

1. I am an Associate Professor at Lund University, Sweden, and former VP Research and Development of Cartela AB, Sweden.

2. My *curriculum vitae* is provided as Appendix 1.

3. I am the sole inventor of the invention disclosed in US Patent Application No. 10/517,210, which relates to integrin α10 chain and integrin α11 chain and their use as marker of mesenchymal stem cells (MSCs).

4. I have reviewed Office communications issued in connection with US Patent Application No. 10/517,210, including those dated May 23, 2008 ("Office

Action I") and December 31, 2008 ("Office Action II"), and the references cited therein. In the following paragraphs I comment and provide my opinion on certain statements made by the Examiner in these communications.

5. The Examiner stated that the specification is enabling for "a method for identifying an integrin alpha 10 chain expressing mesenchymal stem cells comprising a) providing a sample comprising mesenchymal stem cells, b) contacting the sample with FGF-2, c) contacting the sample in step b) with an antibody which specifically binds integrin alpha 10 chain, d) detecting integrin alpha 10 expression on the cell surface of cells of the sample or intracellular in cells of the sample, e) positively correlating the integrin chain alpha 10 expression detected in step e) with the cells being the alpha 10 expressing mesenchymal stem cells." Office Action II, page 3. The Examiner also stated that Example 3 of the specification only supports FGF-2 treated MSCs. Office Action II, page 2.

6. I note that step b) of the method indicated by the Examiner as being enabled (see 5.) is not part of the claimed methods. The other steps of the method indicated by the Examiner as being enabled are similar to the steps of the methods of the pending claims (see, e.g., amended claim 1 in the Amendment and Reply to Office Action filed concurrently with this declaration).

7. The experiment disclosed in Example 3 analyzed the expression of integrin alpha 10 on bone marrow-derived cells that were cultured first without FGF-2 for about two weeks, and then with or without FGF-2 for an additional two weeks. Integrin alpha 10 expression was analyzed by FACS using a monoclonal antibody against alpha 10. Figure 4 shows results for the FGF-2 treated cells but not for the non-treated cells.

8. My understanding is that the Examiner requires step b) ("contacting the sample with FGF-2") for enablement because Figure 4 only shows test results for the FGF-2 treated cells but not for the non-treated cells.

9. At the time the application was filed, one of ordinary skill in the art knew that FGF-2 treatment could be used to promote the growth of MSCs (see specification, ¶ 184 in US 2005/0221327 A1). One of ordinary skill in the art also knew at the time that FGF-2 treatment was not essential for growth of MSCs (see Barry et al. 1999, *Biochemical and Biophysical Research Communications* 265, 134-139, and Quirici et al., 2002, *Experimental Hemataology* 30:783-791, enclosed). Thus, one of ordinary skill in the art knew that MSCs could be grown with or without FGF-2 treatment, and that FGF-2 treatment could be used to enrich MSCs in a sample by promoting their growth.

10. When I conducted the experiment disclosed in Example 3, I obtained the following results. FACS analysis of the bone marrow-derived cells after the first two weeks of culture without FGF-2 revealed that about 50% of cells expressed integrin alpha 10. After the additional two weeks in culture with or without FGF-2, FACS analysis of the FGF-2 treated cells revealed that 96% of the cells expressed integrin alpha 10 (see Example 3 and Figure 4). Further analysis of the FGF-2 treated, integrin alpha 10 expressing cells showed that they were negative for non-MSC marker CD45 and that they formed colonies typical of MSCs (see Example 3). FACS analysis of the non-treated cells revealed that 55% of the cells expressed integrin alpha 10. Thus, the percentage of cells expressing integrin alpha 10 changed less in the absence of FGF-2 treatment. Further analysis of the non-treated, integrin alpha 10 expressing cells

showed that they were also negative for non-MSC marker CD45 and formed colonies typical of MSCs.

11. At the time of filing the application I believed that showing the results for the FGF-2 treated cells was sufficient because these cells displayed a very high percentage of integrin alpha 10 expression and also displayed other MSC characteristics (see 10.). The results for the non-treated cells were qualitatively similar, but with a lower percentage of integrin alpha 10-expressing MSCs.

12. The results obtained for the non-treated cells (see 10.) have since been confirmed. The results are consistent with those described in Varas et al. (cited by the Examiner in Office Action I; see, e.g., Figure 6) even though the overall percentage of integrin alpha 10 expressing cells may vary somewhat between experiments.

13. Based on the results obtained in the experiment disclosed in Example 3, which were confirmed in follow-up experiments such as those described in Varas et al., I believe that treatment of a sample comprising MSCs with FGF-2 was not necessary to enable one skilled in the art to use the claimed methods. The percentage of integrin alpha 10 expressing MSCs in a sample may have varied depending on whether or not the sample was treated with FGF-2, but nonetheless one skilled in the art would have had no difficulty using the claimed methods either way. Importantly, nothing in the application suggested to one skilled in the art that the claimed methods could only be used with FGF-2 treatment.

14. Based on the above, my opinion is that one of ordinary skill in the art at the time the application was filed would have been able to use the claimed methods,

including methods of using integrin alpha 10 as a marker for MSCs and of identifying MSCs, without a significant amount of experimentation, whether or not the sample comprising MSCs was treated with FGF-2.

15. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 2009-06-09 By: _____
Évy Lundgren-Akerlund

Biochemical and Biophysical Research Communications 265, 134–139 (1999)
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The Monoclonal Antibody SH-2, Raised against Human Mesenchymal Stem Cells, Recognizes an Epitope on Endoglin (CD105)

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Mesenchymal stem cells are multipotent cells resident in the bone marrow throughout adulthood which have the capacity to differentiate into cartilage, bone, fat, muscle, and tendon. A number of monoclonal antibodies raised against human MSCs have been shown to react with surface antigens on these cells *in vitro*. A protein of molecular mass 92 kDa was immunoprecipitated using the SH-2 monoclonal antibody. This was purified and identified by peptide sequencing analysis and mass spectrometry as endoglin (CD105), the TGF-β receptor III present on endothelial cells, syncytiotrophoblasts, macrophages, and connective tissue stromal cells. Endoglin on MSCs potentially plays a role in TGF-β signalling in the control of chondrogenic differentiation of MSCs and also in mediating interactions between MSCs and haematopoietic cells in the bone marrow microenvironment. © 1999 Academic Press

Mesenchymal stem cells (MSCs) are resident in the bone marrow stroma throughout adulthood. These cells have a multipotent capacity and can differentiate along a number of well characterized cell lineages including cartilage (1, 2) bone (3), adipose tissue (4), tendon (5) and muscle (6). MSCs have an adherent, fibroblastic phenotype and can be expanded in monolayer culture through many generations. The cells are easily isolated from bone marrow aspirates and, because of their multilineage potential, present exciting opportunities for cell-based therapeutic applications. In fact, therapeutic modalities have been described for the use of MSCs in cartilage (7), bone (8), tendon (5), and bone marrow stroma (9) regeneration and for the treatment of individuals with osteogenesis imperfecta (10).

Several monoclonal antibodies have been used as reagents in the isolation and characterization of MSCs. In general these have been raised against intact human MSCs (11, 12). Recently, one of these antibodies, SB-10, was shown to be reactive with an antigen present on undifferentiated MSCs which disappeared once the cells embarked upon an osteogenic pathway and began to express cell surface alkaline phosphatase. The specific SB-10 antigen was identified as Activated Leukocyte-Cell Adhesion Molecule (ALCAM, 13), which may play a role in the progression of osteogenic differentiation, although the precise mechanism remains to be elucidated (13). Other antibodies that have been described include SH-2, 3 and 4, also raised against human MSCs (12). These antibodies recognized epitopes present on the surface of MSCs and not on haematopoietic cells, and the antigens disappeared upon osteogenic or stromagenic differentiation. Initial studies suggested that each of the antibodies recognized a distinct antigen and that they might find use as reagents for the selection or screening of MSC populations isolated from bone marrow. Since the antigens disappeared upon induction of osteogenesis it also appeared that they would potentially cast light on the regulation of osteogenesis (12). To explore further the role which the SH-2 antigen might play in the biological control of MSC differentiation, and to determine its applicability as a cell surface marker for MSCs, we sought to determine its identity. A recent report described the immunoprecipitation of a glycoprotein of approximate mass 90 kDa from human MSCs (14). In the present study we describe the isolation and purification of the SH-2 antigen from human MSCs and its identification as the TGF-β receptor endoglin (CD105). The expression of endoglin by MSCs and by other cell types is described as well as its potential role in the control of stem cell differentiation.

Abbreviations used: MSC, mesenchymal stem cell; TGF, transforming growth factor; ALCAM, Activated Leukocyte-Cell Adhesion Molecule; MALDI-TOF, matrix-assisted laser desorption time-of-flight.

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METHODS

Isolation of mesenchymal stem cells. Samples of bone marrow were taken from the iliac crest of normal healthy adult volunteers and were processed as described (4, 15). Briefly, a sample of 20–40 ml marrow was collected in a syringe containing 6000 units of heparin. The sample was washed with phosphate-buffered saline and the cellular fraction was recovered by centrifugation. The cells were loaded onto a Percoll layer of density 1.073 g/ml and centrifuged at 1100*g* for 30 min. The nucleated cells were collected from the Percoll-cell layer interface and plated at 200,000 cells/cm^2. The cells were cultured in Dulbecco's modified Eagle's medium (low glucose) containing 10% fetal bovine serum. Medium was changed initially after 24 h and then every third day. MSCs were subcultured at 10 to 14 days by treatment with trypsin and EDTA and reseeded into T-185 flasks. The cells were grown to 80–100% confluence in monolayers at passage 2, 3, or 4. A total of 5×10^6 cells cultured from 8 donors (average age 27.5 y, range 19–35 y) was used for this study.

Immunoprecipitation. The SH-2 antibody used in this study was purified by chromatography on protein G–Sepharose. Immunoprecipitation of the antigen was carried out as follows: Cells in T-185 flasks were washed 3 times with 20 ml cold Tyrode's buffer and the washings were discarded. 15 ml antibody solution containing 1 μg/ml in Tyrode's buffer was added to each flask and incubated at 4°C for 5 h with shaking. The antibody solution was removed and discarded and the cells were washed again with 3 ml Tyrode's buffer which was again discarded. 10 ml fresh Tyrode's buffer was added and the cells were scraped and transferred to a conical tube. The flask was washed with a further 10 ml Tyrode's buffer which was added to the cell fraction. The cells were centrifuged at 2000*g* for 10 min and the supernatant was discarded. Lysis buffer (0.5% CHAPS in 20 mM Tris–Cl, pH 8.0 containing 0.14 M NaCl and 10 mM PMSF) was added at a ratio of 1 ml buffer per 3×10^7 cells. The lysate was incubated on ice for 45 min with regular agitation, then centrifuged at 12,000*g* for 30 min. The supernatant was removed and 550 μl of a 50% slurry of protein G Sepharose (Boehringer) equilibrated in lysis buffer without PMSF was added. The Protein G Sepharose-cell lysate was incubated with rocking at 4°C overnight, centrifuged briefly, washed with lysis buffer, and then centrifuged again. 200 μl SDS–PAGE sample buffer containing 2-mercaptoethanol was added and the material was boiled for 5 min, centrifuged and the supernatant was removed. This step was repeated once and the supernatants were combined. The supernatant containing the solubilized antigen-antibody complex was subjected to electrophoresis on a 12.5% SDS polyacrylamide gel. The gel was stained for 30–60 min with Coomassie blue G and destained in 10% methanol/10% acetic acid until bands were visible. At this point the gel was washed with water and stained bands were excised and stored at −20°C until further use.

In-gel tryptic digestion. Excised gel bands were cut into 1- to 2-mm pieces with a scalpel and incubated for 10 min at room temperature with 50% methanol. This washing procedure was repeated twice. All of the collected gel pieces were combined, dried on a Speedvac and then rehydrated by adding 400 μl 0.1 M Tris–Cl, pH 8.5/0.025% Tween 20 containing 0.5 μg/ml trypsin. After 2 h a further 0.5 ml of buffer was added and digestion was continued overnight at 37°C. As a control, an equal amount of unstained polyacrylamide from the same gel was treated with trypsin in an identical manner. The polyacrylamide pieces were centrifuged and the supernatant transferred to siliconized tubes. 0.5 ml 50% acetonitrile/0.1% trifluoroacetic acid was added to the gel pieces and incubated 1 h at 4°C. This procedure was repeated. The combined supernatants were pooled, dissolved in 0.1% trifluoroacetic acid, and used for peptide mapping. HPLC separation was carried out on a Hewlett-Packard 1090 LC with a 2.1 mm × 250 mm Vydac C18 column. Elution was with a gradient of 0–70% acetonitrile in 95 min and fractions were collected manually.

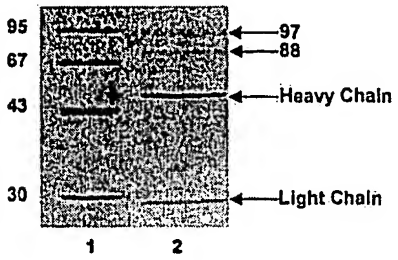

FIG. 1. SDS polyacrylamide gel electrophoresis of cell surface protein on human MSCs immunoprecipitated with SH-2 antibody. Lane 1 shows molecular weight standards and lane 2 shows immunoprecipitated proteins from a solubilized membrane fraction. The numbers on the left refer to the mass of molecular weight standards and on the right to the estimated mass of the immunoprecipitated proteins. The 92-kDa protein band was excised and analyzed further. The heavy and light chains of the antibody are indicated.

Sequence analysis and mass spectrometry. N-terminal sequence analysis of collected peptides was carried out using a Hewlett-Packard G1000A Protein Sequencer and mass spectrometric measurements were taken with a Hewlett-Packard G2026A matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometer. 95% of each fraction eluting from the HPLC was used for sequencing and 5% for MALDI-TOF. For MALDI analysis peptides were mixed with sinapinic acid solution (Hewlett Packard Co., Palo Alto, CA) to achieve a protein concentration of 1–10 pmol/μl. An aliquot (0.8 μl) of this solution was dried on the metal target, forming co-crystals of matrix and protein.

Immunocytochemistry. Embryonic human tissues were obtained from the Central Laboratory for Human Embryology, Department of Paediatrics, University of Washington, Seattle, Washington. Frozen sections of the femur and the hand were taken from tissue of estimated gestational age 84 days. The dissected tissue was placed in Tissue-Tek O.C.T., snap-frozen in a hexane/dry ice bath and cut into 7-μm sections using a cryostat at −29°C. The sections were mounted on Snowcoat glass slides (Surgipath, Richmond, IL) and air-dried at room temperature for 1 h and then stored at −80°C until stained. Staining was with fluorescein-conjugated SH-2 antibody at 10 μg/ml. Incubation was for 2 h at room temperature. Counterstaining was with propidium iodide (Molecular Probes), dissolved at 2 μg/ml. Stained sections were viewed on a Nikon Eclipse TE300 confocal microscope with excitation at 488 nm and detection at 515 and 565 nm.

RESULTS

Figure 1 shows a typical SDS PAGE gel with 2 stained bands of approximate molecular weight 92 and 80 kDa. Both bands were excised but only the 92-kDa material was used for subsequent analysis. Other bands corresponding to the IgG heavy and light chains were also evident on the gel. Figure 2A shows the tryptic map obtained after in-gel digestion of the 92 kDa band along with a control chromatogram (Fig. 2B). Collected fractions (marked with an asterisk) were used for N-terminal sequence analysis (Table I) and MALDI-TOF (Table II). A total of 12 collected HPLC fractions derived from the tryptic digest was used for sequence analysis. In eight of these sequence data were obtained and in the remaining four there was no de-

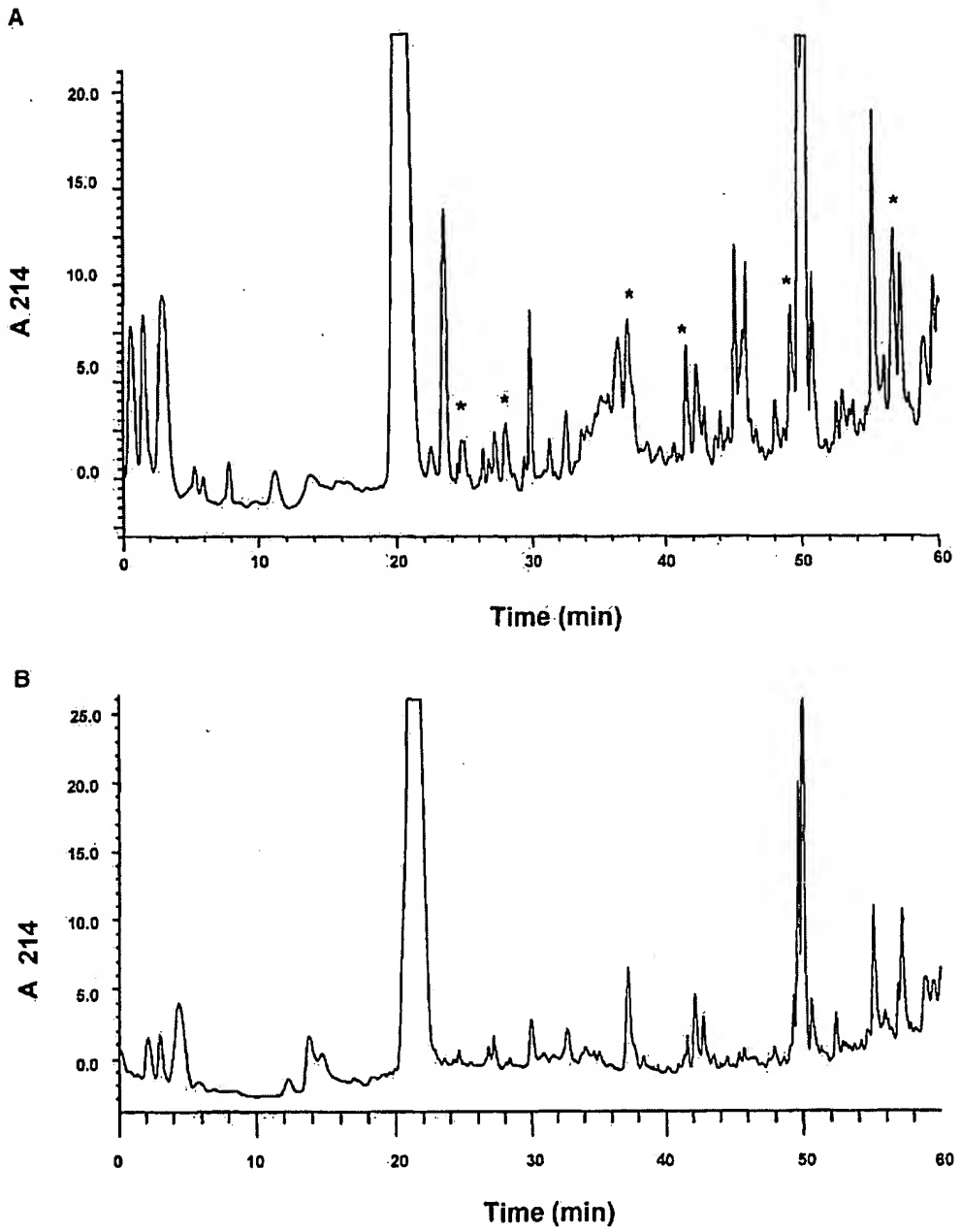

FIG. 2. Separation by reverse-phase HPLC of tryptic peptides derived from digestion in situ of the 92 kDa protein immunoprecipitated with the SH-2 antibody. Peptides were applied to a 2.1 mm × 250 mm Vydac C18 column and eluted with a gradient of 0–70% acetonitrile in 95 min. Separation of the tryptic digest is shown (A) and a control extract taken from an unstained portion of the gel (B).

tectable sequence (Table I). In some fractions there were several detectable sequences. The peptide sequence information obtained was used to search the Genbank database for alignments using the Blitz search routine (16) and all of these showed identity with the human endoglin sequence (17). A total of 12 peptide sequences was determined and aligned with the endoglin sequence (Table I). Seven of the collected HPLC fractions were analyzed by MALDI-TOF (Table II) and in two fractions, those eluting at 35.12 and 41.46 min, several peptide masses were detected. For two of these peptide masses, 2932.8 and 2164.6 Da, there were several possible assignments, all of which are shown in Table II. In a number of cases the mea-

TABLE I

Sequence Information Obtained from Peptides Derived from a Tryptic Digest of SH-2 Antigen

Fraction number	Retention time (min)	Sequence[a]
7	24.71	(210)VLPGHSAGPR
10	28.00	(187)TPALVR
		(204)EAHILR
		(268)IFPEXX
11	29.82	No sequence
13	32.51	(13)ETVHCDXQPVXP
15	35.00	(531)TGTLSX
		(387)GDKFV
		(611)EPVVAVAAP
17	37.12	(13)ETVHXDLQPVGXERGXVTY
21	43.99	(131)TQILEW
22	45.04	No sequence
23	45.81	No sequence
25	49.07	(276)GFKLPDTPQGGLLGEA
32	56.58	(131)TQILEWAAERGPITSAAELN
35	59.55	No sequence

[a] The number in parentheses refers to the residue number that begins the aligned sequence of human endoglin (see Fig. 2).

sured mass suggested an acrylamide or phosphate adduct, as indicated. The mass data obtained were used to search the Genbank database utilizing the mass fitting software MS-FIT (18) and the results of this search indicated that all of the peptides for which definitive mass data were obtained were derived from human endoglin. A total of 12 peptides was identified by MALDI-TOF and these could be aligned with the human endoglin sequence when the measured masses were corrected for the presence of acrylamide or phosphate adducts (Table II). When these were taken into account the measured and calculated peptide masses were comparable within a low level of error (0.71 ± 1.25).

These results all indicated that the SH-2 antigen present on the surface of human MSCs was in fact human endoglin or CD105. Endoglin is a dimeric glycoprotein present on human vascular endothelium, with monomeric mass of 95 kDa based on SDS-PAGE analysis (17). It is a component of the TGF-β signalling system (19), acting as a type III receptor. It has also been detected in bone marrow stroma (20). There is an alternatively spliced variant of human endoglin, referred to as endoglin-S (21), which lacks a 33-residue cytoplasmic tail. This is likely to be the 80 kDa species that we observed on SDS PAGE (Fig. 1).

Because endoglin is an abundant protein associated with endothelial tissues we looked for reactivity of the SH-2 antibody towards different cell types throughout several embryonic tissues. The purpose of this was to compare the reactivity of cells within the bone marrow compartment with cells in endothelial and other tissues. The SH-2 antibody was previously reported to react with fibroblastic cells within the marrow stromal matrix, and to show no reactivity with osteoblasts, chondrocytes or skeletal muscle (12). A more recent assessment of reactivity towards different cells types indicated the presence of the antigen on vascular cells of developing dermis (14).

Fixed specimens of human fetal tissue of estimated gestational age 84 days were probed for reactivity with the antibody and the results are shown in Fig. 3. Positive antibody staining was detected within the bone marrow compartment of the femur and also in the periosteal layer (Figs. 3A and 3B). In the knee joint there was no apparent staining associated with cartilage chondrocytes or with meniscal cells, but positive staining was seen in the cells surrounding the developing vasculature in the meniscus (Fig. 3C). In the soft tissue of the hand (Figs. 3D and 3E) staining was

TABLE II

MALDI-TOF Analysis of Peptides Collected from a Tryptic Digest of the Antigen Immunoprecipitated with SH-2 Monoclonal Antibody

Fraction	Retention time (min)	Measured *m/z*	Calculated *m/z*	%Error	Sequence	Position	Modification
7	24.71	990.7	990.1	0.06	VLPGHSAGPR	210–219	
13	32.51	1651.9	1651.9	0.0	ETVHCDLQPVGPER	1–14	Acryl
15	35.00	1418.5	1418.7	0.01	TGTLSCTVALRPK	531–543	Acryl
17	35.12	1700.3	1699.9	0.02	GFKLPDTPQGLLGEAR	276–291	
		2932.8	2931.4	0.05	MLNA. . . CGGR	292–320	
			2928.3	0.15	ETVH. . .ERGE. . .QVSK	1–26	Acryl
19	41.46	1899.4	1899.1	0.02	AAKG. . .GDPR	498–516	
		2164.6	2163.5	0.05	NIRG. . .GEAR	273–291	Phosphate
			2163.4	0.06	TVFM. . .CTSK	554–572	Phosphate
22	45.04	2319.0	2319.6		EAHI. . .VTVK	204–224	Phosphate
32	56.58	3094.1	3094.5		TQIL. . .ERGP. . .ILLR	131–158	

Note. The mass search program MS-Fit (18) was used to search the Genbank database for identity. Two collected fractions, 17 and 19, gave spectra with two mass signals and the measured masses matched the calculated masses of several tryptic peptides derived from endoglin. All of the possible matches are included.

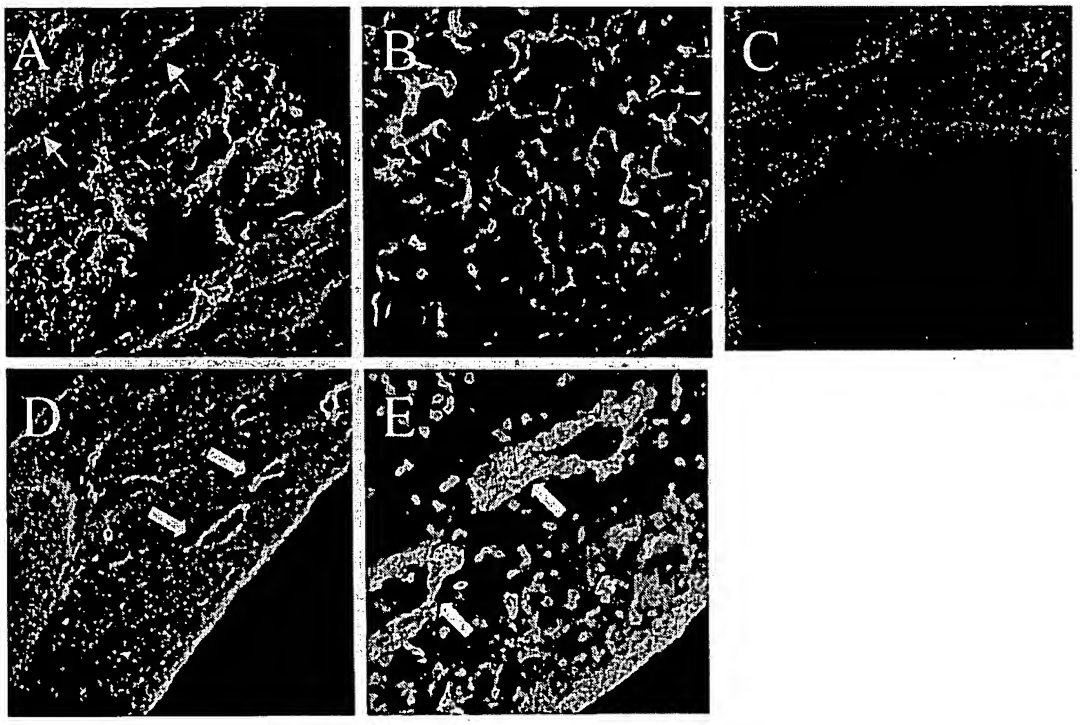

FIG. 3. SH-2 staining in human fetal tissues. Positive antibody staining was detected within the bone marrow compartment of the femur shown at 10× original magnification (A) and 40× original magnification (B). In (A) antibody staining was also evident in the periosteal layer. In the knee joint (C) there was no apparent staining associated with chondrocytes or with meniscal cells, but positive staining was seen in the cells surrounding the developing vasculature in the meniscus (arrow). In the soft tissue of the hand (D) staining was evident in the endothelial cells that surround developing blood vessels (arrows). These are shown in higher magnification (E) to show the specific staining of endothelial cells.

evident in the endothelial cells on developing blood vessels.

DISCUSSION

In this study we have identified the SH-2 antigen as endoglin (CD105), a TGF-β type III receptor that is present on many cell types. The identification was made by determination of the sequence and mass of a number of peptides derived from the protein that was specifically immunoprecipitated from human MSCs with the antibody.

The monomeric mass of the core protein of endoglin is 68,000 Da based on amino acid sequence data. The additional mass of the mature protein (97 Da) can be accounted for by N- and O-glycosylation. In fact endoglin contains 4 N-linkage sites and a putative O-glycosylation domain, all of these sites occurring on the extracellular portion of the molecule (17). Gouges and Letarte (22) showed that endoglin from the pre-B leukemic HOON cell line was reduced by 20,000 Da after treatment with *N*-glycosidase or endoglycosidase F and by 15,000 Da following *O*-glycanase and neuraminidase treatment. Our results are consistent with those measurements. Fleming *et al.* (14) showed a similar reduction in size after treatment with N-glycanase but not with *O*-glycanase.

The role of endoglin in signal transduction has been well characterized. Cheifetz *et al.* (19) showed that endoglin binds TGF-β1 and TGF-β3 with high affinity but not TGF-β2. In this sense endoglin is distinguished from betaglycan which binds to all three isoforms of TGF-β (19). It appears to form a complex with TGF-β receptors I and II (23) and presumably can play a role similar to that played by betaglycan (24) in regulating access to TGF-β. The key role played by endoglin in endothelial cell interactions or vascular development is underlined by the finding that hereditary haemorrhagic telangiectasia, an autosomal dominant disorder characterized by widespread vascular failure and haemorrhage, is caused by a mutation in the endoglin gene (25).

Endoglin may play a role in mediating interactions between haematopoietic and mesenchymal stem cells in the marrow. Since endoglin is expressed on early B-lineage precursor cells (CD19+ and CD34+), proerythroblasts (CD71+ and glycophorin A+) in fetal bone marrow (26), it is possible that interactions are

mediated via the cell-binding domains. Another possible role for endoglin on MSCs may be in mediating TGF-β signalling during chondrogenic differentiation. All TGFβ isoforms (2, 27) are capable of inducing MSCs from human and other species along the chondrogenic pathway. TGFβ-2 is also capable of initiating chondrogenesis but does not bind to endoglin, suggesting that other mechanisms are also possible.

ACKNOWLEDGMENT

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Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies

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Objective. **Mesenchymal stem cells (MSCs) are a population of multipotent cells that can proliferate and differentiate into multiple mesodermal tissues. We previously reported that monoclonal antibodies to the low-affinity nerve growth factor receptor (α-LNGFR) stain bone marrow (BM) mesenchymal cells. We now show that LNGFR antibodies label primitive MSCs with high specificity and purity in adult BM, and compare these cells to those isolated by plastic adherence (PA) and CD45⁻anti-glycophorin A⁻ selection.**

Materials and Methods. **Low-density mononuclear cells (LD-MNCs) from normal BM were separated by PA or immunomagnetic selection for NGFR⁺ or CD45⁻α-glycophorin A⁻ cells. The three fractions were grown in Iscove's modified Dulbecco medium + 20% fetal bovine serum ± basic fibroblast growth factor (bFGF) in order to assess their proliferative capacity and evaluate their phenotype during culture. The clonogenic potential of the MSCs was assessed using a colony-forming unit fibroblast (CFU-F) assay, whereas multipotential differentiation was determined after culture in adipocytic and osteoblastic conditioned media.**

Results. **The NGFR⁺ mesenchymal cells grown without growth factors showed persistent NGFR expression (rapidly down-regulated after the addition of bFGF) and persistent CFU-F activity. The NGFR⁺ fractions were rich in clonogenic precursors: CFU-F median frequency was 1584/1 × 10^5 cells (range 325–13,793) in the NGFR⁺ cells and 35/1 × 10^6 cells (range 27–112) in the LD-MNCs. The NGFR⁻ fraction never showed any residual CFU-F activity. Compared with the other two fractions, the NGFR⁺ cells (± bFGF) showed a 1 to 3 log greater expansion in the number of fibroblastic cells and a greater capacity to give rise to adipocyte colonies and induce osteoblastic differentiation, and they had similar effects in supporting the growth of hematopoietic precursors.**

Conclusion. **The data suggest that positive selection using low-affinity NGFR antibodies makes it possible to obtain homogeneous multipotent MSCs.** © **2002 International Society for Experimental Hematology. Published by Elsevier Science Inc.**

The complex process of hemopoiesis is regulated by the bone marrow (BM) microenvironment, a heterogeneous tissue consisting of macrophages, endothelial cells, adipocytes, and fibroblasts. The precursors of nonhematopoietic cells in this tissue originally were referred to as plastic-adherent (PA) cells or colony-forming units fibroblasts (CFU-F) [1,2] and more recently have been defined as mesenchymal stem cells (MSCs) or mesenchymal progenitor cells [3] because they can differentiate into a variety of nonhematopoietic cells. Since the initial report by Friedenstein et al [4], a large number of reports have described the functional and phenotypical characteristics of these cells [2], but it is only recently that multiple lineages (osteogenic, chondrogenic, and adipogenic) have been clonally derived from a single MSC [5]. These cells also are capable of expanding while still showing a stable phenotype, and thus open the way to the transplantation of nonhematopoietic stem cells with the aim of reconstituting a variety of tissues.

Despite extensive functional characterization, the MSC phenotype has not been clearly described. It is known that

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they are CD34$^-$ and CD45$^-$, and they express a variety of the antigens shared by hematopoietic and endothelial cells (i.e., SH2) [6]. The Stro-1 antibody, which binds to all of the cells associated with CFU-F activity of adult human BM [7–9], also binds to glycophorin A$^+$ and CD19$^+$ cells. The lack of a specific marker of BM MSCs has led some authors to explore negative selection procedures, such as those that involve the purification of CD45$^-$glycophorin A$^-$ cells [10].

The staining of mesenchymal cells (MCs) by anti-nerve growth factor receptor (anti-NGFR) antibodies was first reported by Thomson et al. [11–13], but the NGFR$^+$ BM stroma was not extensively characterized until 1993 [14]. These antibodies label the majority of reticulin$^+$ and collagen III$^+$ BM stromal cells, but not hematopoietic or endothelial cells. This antigen also has been described on the earliest component of BM stroma in developing human fetus epiphyseal bone [15,16], in the BM cavity before the appearance of BM activity, and in 7 to 11% of cells from the adherent layer of long-term BM cultures, thus suggesting that NGFR antibodies also may stain primitive MCs.

We here show that LNGFR antibodies do label adult BM MSCs with high specificity and purity, and suggest that positive selection using these antibodies may become the method of choice for obtaining multipotent cells.

Materials and methods

Mesenchymal cells

After obtaining informed consent, an average 30 mL of BM from 11 healthy allogeneic BM transplantation donors was collected in heparinized tubes and layered on a Ficoll-Paque gradient (specific gravity 1.077 g/mL; Nycomed Pharma AS, Oslo, Norway). The low-density mononuclear cells (LD-MNCs) were washed twice in Hank's balanced salt solution and resuspended in Iscove's modified Dulbecco medium (IMDM) (BioWhittaker, Caravaggio, Italy) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Sigma-Aldrich, Milan, Italy).

Isolation and culture of BM MCs

Plastic adherence. The MSCs were purified from normal BM according to Friedenstein et al. [4,5]. In brief, 2×10^7 BM mononuclear cells were plated in a 25-cm^2 tissue culture flask (Costar, Cambridge, UK) in IMDM supplemented with 20% FBS and antibiotics, and incubated at 37°C with 5% fully humidified CO_2. After 3 to 4 days, the nonadherent cells were removed and the medium was replaced. The cultures were refed weekly and grew to confluency within 3 to 4 weeks, when they were processed by trypsinization (0.25% trypsin and 1 mM EDTA for 5 min at 37°C) and reexpanded to confluency. After a second passage, the cells were counted and assessed for viability by means of Trypan blue dye exclusion.

Purification of CD45$^-$α-glycophorin A$^-$ MCs. The MCs were isolated by negative selection of CD45$^-$α-glycophorin A$^-$ BM cells [10]. In brief, the BM LD-MNCs were incubated with monoclonal antibodies (mAbs) against the human leukocyte common antigen CD45 and human glycophorin A for 15 minutes at 4°C, rinsed, incubated with anti-IgG1 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 minutes at 4°C, and placed on a column in a "midiMACS" cell separator (Miltenyi Biotec). The unlabeled cells (negative fraction) were eluted from the column and subjected to a second step of purification. The cells then were counted, assessed for viability, and their purity determined using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Positive selection of α-NGFR$^+$ MSCs. The LD-MNCs were incubated with Me20.4 mAb labeling the LNGF receptor (or p75^{NGFR}) (Upstate Biotechnology, Lake Placid NY, USA) for 15 minutes at 4°C, rinsed, incubated with anti-IgG1 immunomagnetic beads for 15 minutes at 4°C, and placed on a midiMACS column. The positive fraction was subjected to a second separation step. The cells then were counted, assessed for viability, and their purity determined by flow cytometry using a α-LNGFR-phycoerythrin (PE) antibody reacting with a different epitope (clone C-40-1457; BD PharMingen).

Immunocytochemistry and flow cytometry

Primary antibodies used. The following primary antibodies were used: mAbs against LNGFR (Me20.4) (Upstate Biotechnology) and R-PE-conjugated LNGFR (C40-1457; BD PharMingen), the monoclonal mouse antifibroblast TE7 (Harlan Sera Lab, Oxford, UK), SH2 (CD105 SN6; Serotec, Oxford, UK), CD34, CD34 HPCA-2/fluorescein isothiocyanate (FITC) and PE, CD14 (LeuM3), and CD14 FITC (all by Becton-Dickinson), Thy1 (CD90 5E10; BD PharMingen), α-glycophorin A (JC159) and CD45 (T29/33) (Dako, Milan, Italy), and AC133/2 and AC133/2-PE (CD133) (Miltenyi Biotec).

Flow cytometry. A total of 2.5×10^5 cells were resuspended in 50 μL of RPMI containing 20% FBS and the selected primary mAbs for 30 minutes at 4°C. In the case of primary unconjugated mAbs, the cells were first incubated with the primary antibody (30 min, 4°C), washed twice, and incubated with FITC-conjugated goat anti-mouse antibody (Dako) for 30 minutes at 4°C. Cell fluorescence was evaluated by flow cytometry using a FACSCalibur instrument and the data analyzed using CellQuest software (BD).

Immunocytochemistry

Immunocytochemistry was generally preferred for the phenotype analysis of adherent MCs. After trypsin digestion, the cells were cytocentrifuged onto glass slides (Cytospin II, Shandon), and the presence of surface markers was detected using the alkaline phosphatase-antialkaline phosphatase (APAAP) technique as previously described [17].

MSC progenitor assays

Colony-forming unit-fibroblasts. The details of this procedure have been previously described [18]. In brief, 1×10^4 NGFR$^+$ cells, 1×10^6 LD-MNCs, and 1×10^7 NGFR$^-$ cells were resuspended in IMDM and 20% FBS in T-25 flasks, and incubated in a fully humidified atmosphere with 5% CO_2 at 37°C for 5 days. During the fifth day of culture, all of the medium was removed and replaced by fresh medium. After 9 days of culture, the flasks were washed twice, fixed with methanol, and stained with Crystal violet. The fibroblast colonies were counted using an inverted microscope at 25×. Cell clusters consisting of at least 50 fibroblasts were scored as a CFU-F colony.

CFU-F in limiting dilution assay. The frequency of CFU-F in LD-MNCs and the NGFR$^+$ and NGFR$^-$ fractions was determined by

means of a limiting dilution assay. The cells from the different fractions were resuspended in IMDM and 20% FBS, and seeded in 96-well microtiter plates in five dilution steps (16 replicates for each dilution): 60/120/180/240/300 cells/well for the $NGFR^+$ fraction and 3000/6000/9000/12,000/15,000 cells/well for the $NGFR^-$ fraction and the LD-MNCs. During the fifth day of culture, half of the medium was removed and replaced by fresh medium. After 9 days, the flasks were rinsed, fixed with methanol, and stained with Crystal violet. The incidence of negative wells was determined and the frequency of colonies evaluated by means of the Poisson statistic [19].

MSC cultures

After separation and until senescence, 5×10^4 $LNGFR^+$ cells and 3×10^5 $CD45^-\alpha$-glycophorin A^- and PA cells were maintained and expanded in IMDM supplemented with 20% FBS, with weekly refeeds. At confluency, the cells were trypsinized, counted to assess their fold increase, tested for CFU-F efficiency, centrifuged onto glass slides for immunocytochemical analysis, and 10% of the cells reexpanded to confluency.

Functional assays

Fibroblastic differentiation of MSCs. To induce fibroblastic differentiation, 5×10^4 $LNGFR^+$ cells and 3×10^5 $CD45^-\alpha$-glycophorin A^- and PA cells were incubated in IMDM supplemented with 20% FBS and 10 ng/mL basic fibroblast growth factor (bFGF; Peprotech, London, UK) in T-25 flasks. The medium and the growth factor were replaced weekly. At confluency, the cells were trypsinized, counted to assess their fold increase and population doublings, centrifuged onto glass slides for immunocytochemical analysis, and 10% of the cells reexpanded. This process was repeated at each confluency, up to senescence.

Adipogenic differentiation of MSCs. To induce adipocyte differentiation, 5×10^4 MSCs from the different isolation methods were cultured in Dulbecco's modified Eagle medium with 4.5 g/L glucose supplemented with 10% FBS (BioWhittaker), 0.5 mM isobutyl-methylxanthine, 200 μM indomethacin (Sigma), 10^{-6} M dexamethasone (Sigma), and 10 μg/mL insulin in T-25 flasks, with weekly refeeds [5]. The cells containing lipid vacuoles were observed within 2 to 3 weeks. At the end of the culture, the cells were fixed in 10% formalin for 10 minutes and stained with fresh oil red-O solution (Sigma). The total number of oil red-positive adipocytes or adipocyte colonies in each flask was counted.

Osteogenic differentiation of MSCs. To induce osteogenic differentiation, 5×10^4 separated cells were plated in six-well microplates (Costar) in α-MEM supplemented with 10% FBS (BioWhittaker), 10 mM β-glycerolphosphate (Sigma), 0.2 mM ascorbic acid (Sigma), and 10^{-8}M dexamethasone, and cultured for 21 to 28 days with weekly refeeds [20]. To demonstrate osteogenic differentiation, the cultures were washed with phosphate-buffered saline, fixed with ice-cold ethanol 70% for 1 hour and stained with alizarin red S (40 mM, pH 4.1 for 10 min) (Sigma) in order to assess calcium accumulation [21]. Mineralization was assayed by examining the total area of the confluent culture and scoring the areas of mineralization as a percentage of the total.

Cocultivation of $CD34^+$ hematopoietic progenitors with fibroblastic layers derived from $NGFR^+$ cells

Coculture assessments. The fibroblasts from $NGFR^+$ cultures were treated with trypsin, inactivated with mitomycin C (25μg/mL mitomycin C/1 $\times$ 10^6 cells for 30 min at 37°C), and 3.6×10^3 cells/well were seeded in six-well microplates (Costar). A total of 100,000 normal peripheral blood $CD34^+$ cells from granulocyte colony-stimulating factor-mobilized donors were plated in direct contact with the stromal layers or in a transwell insert with a 0.4-μm microporous filter membrane (Falcon; Becton-Dickinson) placed above the feeder layer. The cultures were maintained for 3 weeks in a humidified atmosphere at 37°C and 5% CO_2, with weekly media changes without a demipopulation of the culture supernatant. The cells harvested from the supernatant and stroma of the contact and noncontact cultures were counted and replated in methylcellulose in order to determine the number of colony-forming cells.

Progenitor assays. The colony-forming unit granulocyte-macrophage (CFU-GM) and burst-forming unit erythroblast (BFU-E) assays were carried out by plating 5×10^4 cells in a methylcellulose culture medium (MethoCult GF H4434; Stem Cell Technologies, Vancouver, Canada). Triplicate dishes were incubated at 37°C and 5% CO_2 in a fully humidified atmosphere. After 14 days of culture, aggregates consisting of at least 40 cells were scored as colonies and counted.

The absolute number of CFU-GM and BFU-E colonies was obtained by normalization to the total number of cells harvested under the different culture conditions.

Results

Enrichment of MSCs

The mean percentage of $NGFR^+$ cells in the LD-MNC fraction was 2.3 ± 0.8, with a recovery of 0.34% (± 0.3; n = 11) of cells after immunomagnetic separation. The yield of $NGFR^+$ cells after separation (number of positive cells in the final fraction × percentage purity of the $NGFR^+$ cells/initial number of $NGFR^+$ cells) was 12% ± 4%. The purity of separated cells, as determined by flow cytometric analysis, was 90.5% ± 3.5%.

The mean percentage of $CD45^-\alpha$-glycophorin A^- cells in the LD-MNCs was 2.08% ± 0.08%. The purity of the negatively separated cells was 92.7% ± 1.5% as determined by FACS analysis.

MCs were obtained by PA after two steps of trypsinization. The mean percentage of cells with a mesenchymal phenotype ($CD45^-\alpha$-glycophorin A^-) was 98% ± 1%.

Phenotype analysis of $NGFR^+$ MSCs

The $NGFR^+$ fraction consisted of a majority of small round cells (Fig. 1a) rapidly adhering to the plastic (Fig. 1b) and expressing surface markers associated with a primitive phenotype ($CD34^+$ cells: 44.1% ± 45.8%; and $CD133^+$ cells: 49.4 % ± 29.9%), whereas only a minority of cells showed the "putative" mesenchymal markers (SH2, CD90, TE7) (Table 1 and Fig. 2).

CFU-F assays

The clonogenic efficiency of the normal BM samples was highly variable, but the $NGFR^+$ fractions were much richer in clonogenic precursors. The median number of CFU-F

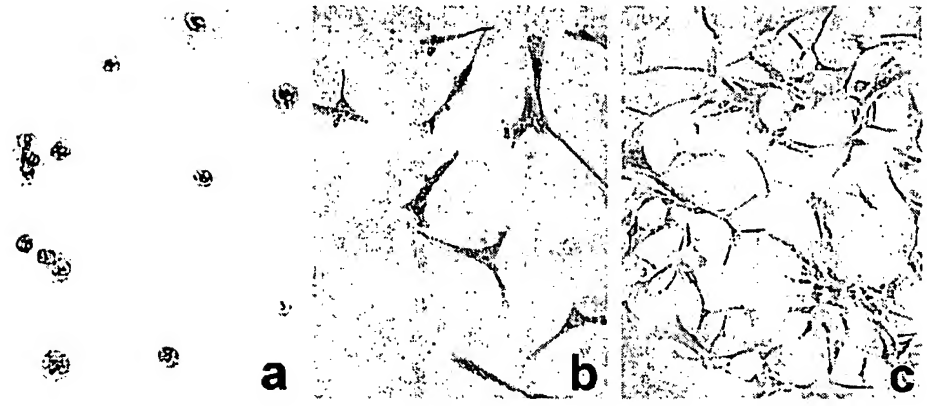

Figure 1. NGFR$^+$ cells when first isolated and after 2 and 15 days of culture. This positively selected population consists of homogeneously small and round cells (a) (Turk staining) that rapidly adhere to the plastic (b) and in the presence of bFGF form a confluent cell layer with a fibroblastic morphology (c).

colonies in 1 × 10^6 seeded cells was 2700 (range 200 to 8700) in the NGFR$^+$ fractions and 44 (range 18–78) in the LD-MNCs. In addition, no residual CFU-F activity was observed in the NGFR$^-$ fractions (Table 2).

These data were further quantitated by seeding the cells from the LD-MNCs and NGFR$^+$ and NGFR$^-$ fractions under limiting dilution assay and determining the CFU-F frequency by means of the Poisson statistic. CFU-F median frequency was 1584 colonies/1 × 10^6 cells (range 325–13,793) in the NGFR$^+$ fractions and 35 (27–112) in the LD-MNCs. The NGFR$^-$ fractions showed no residual CFU-F activity (Table 2).

Maintenance of purified MSCs without growth factor

The NGFR$^+$ cells separated from three different BM samples were maintained in culture for more than 2 months in the presence of IMDM supplemented with 20% FBS, but without the addition of specific growth factors. During culture, the cells expanded over 10^4 cell doublings still retaining their clonogenic capacity, while PA and CD45$^-$α-glycophorin A$^-$ fractions expansion capacity were 2 to 3 logs lower. CFU-F efficiency of the NGFR$^+$ cells, assayed at every trypsinization step of the flask, remained stable throughout the duration of the culture and declined precipitately after 2 months (Fig. 3). These cells were maintained without any lineage-specific growth factor and were driven to mesenchymal multilineage differentiation every 2 weeks for 2 months. Their fibroblastic and osteogenic capacities were maintained throughout the 2 months of culture, while the adipogenic differentiation capacity already disappeared after 2 weeks.

Immunophenotype characterization of MSCs cultured with or without growth factor

The phenotype of the MCs separated using the different methods was analyzed after 2 and 4 weeks of culture in media promoting fibroblastic growth, and the results were compared with those obtained in cultures grown in the absence of growth factor. All of the cells rapidly converted to a mesenchymal phenotype (greater than 90% of CD45$^-$TE7$^+$ cells), but there was a persistent small percentage of CD14$^+$ cells of monocytic origin in the MCs obtained by PA (Table 3).

On the other hand, the cells grown in the absence of growth factors expressed high levels of NGFR (particularly in the NGFR$^+$ fraction), although the receptor was rapidly down-modulated in the presence of bFGF (Table 3).

In both cases, the LNGFR$^+$ cells became CD34/CD133 less than 1% or negative after only 2 weeks in culture.

Functional assays

Fibroblastic differentiation. When grown in the presence of bFGF, the cells formed a confluent cell layer with a fibroblastic morphology (Fig. 1c). The expansion capacity varied widely with samples from different donors, but both cell number fold increase and population doublings were much higher in the NGFR$^+$ than the PA and CD45$^-$α-glycophorin

Table 1. FACS analysis

	NGFR	CD34	CD133	SH2	CD90	TE-7
LD-MNCs	2.3 ± 0.8	0.98 ± 0.5	0.41 ± 0.4	4 ± 1.1	2.3 ± 1.3	1.3 ± 1.8
NGFR$^+$ cells	90.5 ± 3.5	44.1 ± 45.8	49.4 ± 29.9	9.9 ± 10	6.3 ± 9.7	10 ± 14

Percentage ± SD of cells labeled in the bone marrow mononuclear cells (prior to separation) and in the NGFR$^+$ cells.

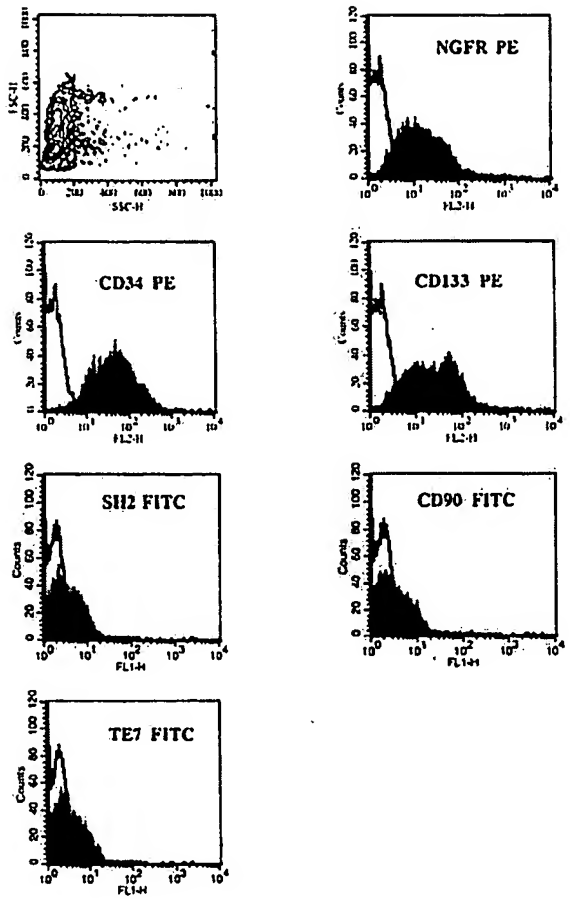

Figure 2. FACS analysis of a NGFR$^+$ separation. The open histograms indicate negative controls. In this experiment, most of the cells are homogeneously small (low side scattering) and coexpress both CD34 and CD133.

A$^-$ cultures, whose expansion rates were not significantly different in the various experiments. After 8 weeks of culture, the expansion rate of the NGFR$^+$ cells was 1 to 3 logs greater than that of the other fractions (Fig. 4).

Table 2. CFU-F activity

	α-NGFR$^+$	α-NGFR$^-$	LD-MNCs
CFU-F* (no. of colonies/1 × 10^6 cells)			
Median	2,700	0	44
Range	200–8,700		18–78
LD assay† (CFU-F frequency/1 × 10^6 cells)			
Median	1,584	0	35
Range	325–13,793		27–112

*Results of nine experiments.
†Results of five experiments.

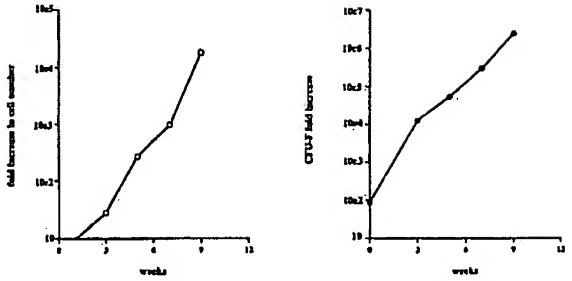

Figure 3. In the absence of specific growth factors, NGFR$^+$ cells grow, expand, and retain their clonogenic capacity for more than 2 months. Results from a representative experiment.

Adipogenic differentiation. When grown in adipogenic medium, the MSCs isolated using the three different methods showed clear-cut differences, as revealed by oil red-O staining. The NGFR$^+$ cells showed an increasing number of adipocyte colonies starting 2 weeks after seeding; the CD45$^-$glycophorin A$^-$ cells only a few colonies or single adipocytes, and the PA cells only differentiated into single adipocytes (Table 4 and Fig. 5).

Osteogenic differentiation. After 3 to 4 weeks of culture, the cells driven to osteogenic differentiation clearly showed an increase in calcium accumulation, as revealed by alizarin red S staining. The NGFR$^+$ cells had a larger mineralized area (% of the total area of the well) than the CD45$^-$α-glycophorin A$^-$ or PA cells: 82 ± 14.3% (mean ± SD of three samples) as against 23 ± 8.5% and 11 ± 6.7% (Fig. 6).

Hematopoietic cell support of NGFR$^+$-derived MC layers
MCs, that were obtained by NGFR$^+$ selection, support hematopoiesis as well as BM stroma from Dexter-type LTBMC. To assess whether "pure" fibroblastic layers modulate hematopoiesis by cell-to-cell interactions, the cocultures were established under contact and noncontact conditions. CFU-GM output was higher in the contact cultures (mean ± SD: 14,170.5 ± 815 vs 11,819.5 ± 675.3), but BFU-E output was higher in the transwell cultures (147.5 ± 37.5 vs 644.5 ± 358.5); however, these differences were not statistically significant (Student's *t*-test) (Fig. 7).

Discussion

MCs, which are marrow-derived progenitor cells characterized by the capacity for self-renewal and differentiation into multiple lineages, including osteoblasts, adipocytes, fibroblasts, and chondrocytes, have been extensively investigated over the last few years. However, there is still a lack of specific cell markers and methods for selectively isolating the more primitive cells. The results of this study demonstrate that the immunomagnetic sorting of BM cells labeled with low-affinity NGFR antibodies allows the selection of phenotypically and functionally homogeneous cells that

Table 3. Immunophenotypic characteristics of MCs obtained with the different methods and grown in the presence or absence (no GFs) of bFGF

	α-NGFR		TE-7		CD45		CD14	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
NGFR$^+$ MCs, no GFs	41 ± 7.7	34 ± 5.4	98 ± 2.5	100	3 ± 2	0	3 ± 1.8	0
NGF-R$^+$ MCs + bFGF	7 ± 9	1.2 ± 1.9	98 ± 3.7	97 ± 5	0.8 ± 0.8	0.2 ± 0.7	0.7 ± 0.9	0.2 ± 0.7
CD45$^-$/glycophorin A$^-$ MCs + bFGF	0.5 ± 0.7	0.3 ± 0.5	95 ± 7	98.3 ± 2.8	2.5 ± 2	1.6 ± 0.8	1.5 ± 2	1.8 ± 0.5
PA MCs, no GFs	4.5 ± 4.9	16.7 ± 15.3	91 ± 12.7	90 ± 0.9	6.5 ± 0.7	9 ± 9.5	8 ± 0	5.7 ± 8.9
PA MCs + bFGF	3.3 ± 4	4 ± 4.2	94 ± 10	99.8 ± 0.5	5.3 ± 4.6	0	4.3 ± 3.7	0

Results are expressed as mean ± SD of five different experiments.

are capable of expansion, self-renewal, and differentiation into multiple MC lineages, and can also support hematopoiesis.

LNGFR expression previously has been described in BM and in other tissues [11,12,14,15]. Anti-NGFR antibodies label the majority of BM stromal cells, i.e., those that are reticulin$^+$, vimentin$^+$, collagen III$^+$, TE7$^+$, and CD90$^+$ [14]. When examined in situ in BM biopsies and spiculae, these cells have long dendrites that make contacts with the abluminal side of sinus endothelial cells and intermingle with islands of developing hematopoietic cells [14,22]. Wilkins and Jones [23] previously attempted to isolate NGFR$^+$ cells from human BM, but their yields were insufficient for cell characterization or to generate successful cultures. By processing a large volume of BM and performing two rounds of immunomagnetic selection, we were able to select a small subpopulation of NGFR$^+$ cells (an average of 10% of the NGFR$^+$ cells in whole BM) that express markers of primitive cells. This positively selected population consists of homogeneously small and round cells, whereas the majority of cells with the spindle-shaped or large flat morphology with a phenotype of more mature fibroblasts [24–26] remain in the columns during immunomagnetic separation. In fact, the unlabeled fractions are always completely depleted of cells with MSC phenotype and CFU-F activity. The immunoselected cells are a highly homogeneous subpopulation of cells with a high proliferative capacity and the potential for multilineage differentiation along the mesenchymal series. Expansion rates of fibroblastic, adipogenic, and osteogenic precursor cells were much higher from NGFR$^+$ cells than those from PA or CD45$^-$α-glycophoryn A$^-$ separations.

A subpopulation of small MSCs characterized by a rapid rate of replication and an enhanced potential for multilineage differentiation was identified recently by Colter et al. [27]. This population, selected by PA and flow sorting on the basis of forward and side light scattering, was CD34$^-$ and CD133$^-$ but Trk$^+$, and may overlap the NGFR$^+$ MSC

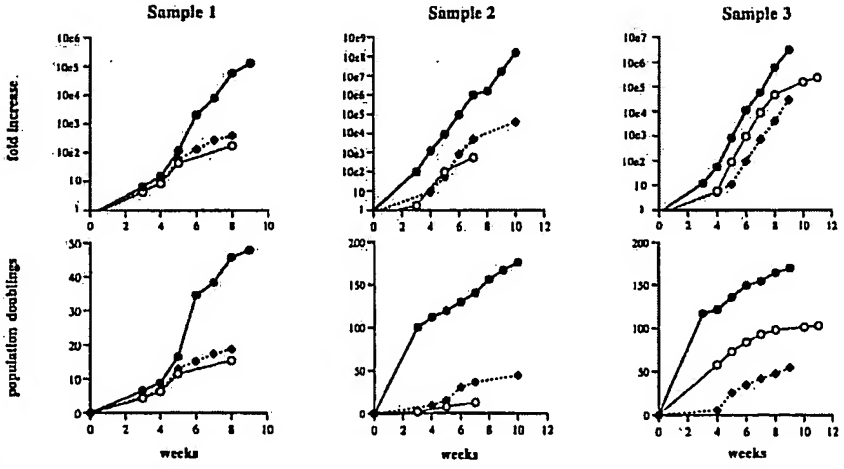

Figure 4. Fold increase (final absolute number of cells obtained when all of the starting cells expanded to senescence, divided by the starting number of cells) (see Materials and methods) and population doublings of MCs driven to expansion and differentiation into fibroblasts. Filled circles: BM cells separated using α-NGFR antibodies were seeded at a concentration of 5 × 10^4 cells in T-25 flasks, grown to confluency, and harvested. The process was repeated until senescence. Open circles: CD45$^-$α-glycophorin A$^-$ BM cells were negatively separated by means of immunomagnetic sorting, seeded at a concentration ranging from 5 × 10^4 to 3 × 10^3 cells in T-25 flasks, and processed as described earlier. Filled diamonds: Plastic-adherent BM cells after two rounds of trypsinization. The cells were seeded at a concentration of 3 × 10^3 cells in T-25 flasks and processed as described earlier. Results of three representative bone marrow samples.

Table 4. Adipocyte differentiation

	NGFR$^+$	CD45$^-$/α$^-$glycophorin A$^-$	PA
1	264 colonies	6 colonies	0 colonies
		480 cells	1564 cells
2	32 colonies	0 colonies	ND
		38 cells	ND
3	552 colonies	1 colony	0 colonies
		156 cells	1875 cells

Absolute no. of colonies or single cells differentiated in adipocytes per T-25 flask (3×10^5 cells seeded) of three different bone marrow samples. ND = not done.

population described here. The absence of primitive stem cell markers in this MSC population may be due to culture conditions, whereas our cells were analyzed immediately after immunoseparation. Nevertheless, a careful comparison of the multipotent characteristics of these two populations may be interesting.

The phenotype of the NGFR$^+$ selected cells is intriguing, because we found no evidence of the presence of a subpopulation coexpressing NGFR and CD34 in a previous in situ study [14]. The poor sensitivity of in situ double labeling immunocytochemical techniques in detecting rare cell populations such as those coexpressing NGFR and CD34 may explain this discrepancy. On the other hand, expression of CD34 has been reported in MSCs directly isolated from BM, although it was rapidly lost after in vitro culture [6,9]. The variable but consistent expression of CD34 on NGFR$^+$ cells separated by immunomagnetic selection was reinforced by our data showing simultaneous expression of CD133, a marker generally associated with a more primitive

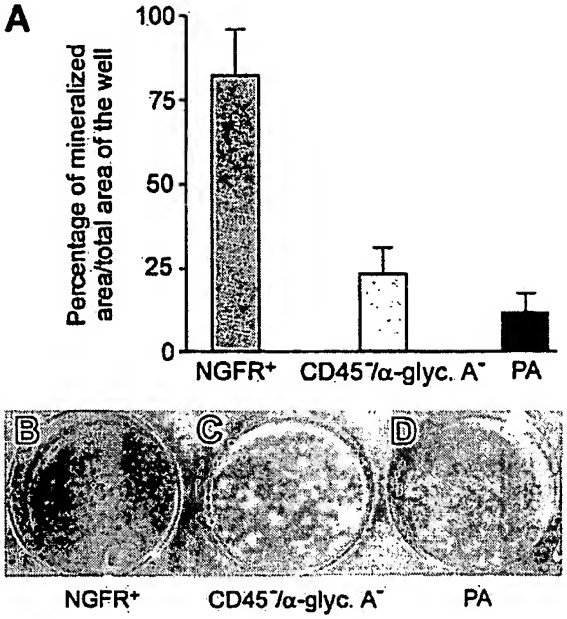

Figure 6. MSCs from the three fractions driven to osteogenic differentiation showed an increase in calcium accumulation, as revealed by alizarin red S staining. (A) Percentage of mineralized area/total area of the well (mean values ± SD of three samples). After 3 to 4 weeks of culture, the NGFR$^+$ cells (B) had a larger mineralized area than the CD45$^-$α-glycophorin A$^-$ (C) or PA cells (D).

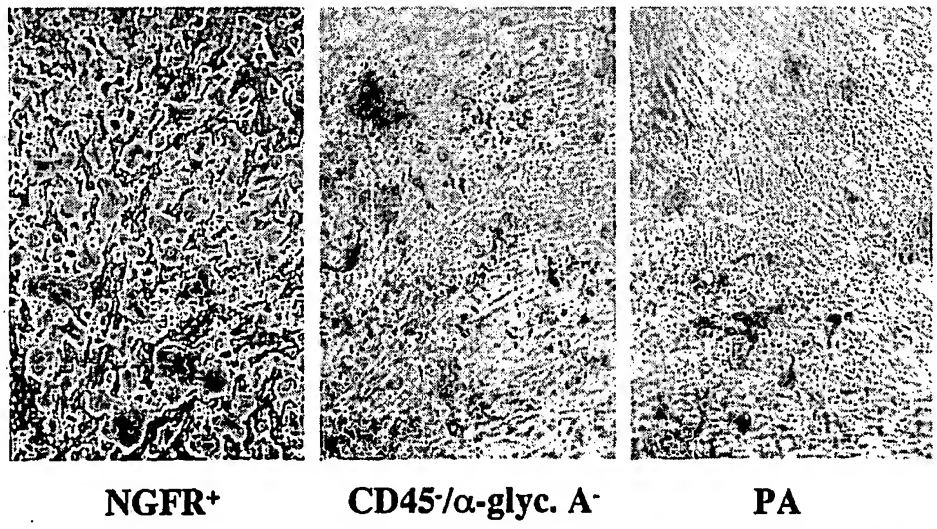

Figure 5. MSCs from NGFR$^+$ (A) or CD45$^-$α-glycophorin A$^-$ (B) immunomagnetic selection and PA (C) were cultured in adipogenic media for 3 to 4 weeks. NGFR$^+$ MSCs driven to adipogenic differentiation gave rise to a high number of adipocyte colonies, whereas CD45$^-$α-glycophorin A$^-$ and PA MSCs gave only a few colonies or single adipocytes, as indicated by the accumulation of lipid vacuoles stained by oil red-O. All fields are at the same magnification.

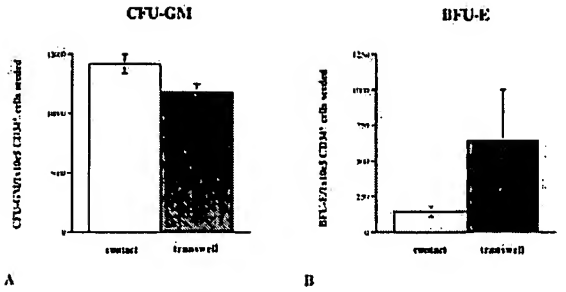

Figure 7. Cocultures of CD34$^+$ cells with fibroblastic layers derived from NGFR$^+$ cells. Immunomagnetically separated CD34$^+$ cells (from granulocyte colony-stimulating factor-mobilized peripheral blood) were placed in direct contact with MCs (contact cultures) or separated by a 0.4-μm microporous transwell membrane (noncontact cultures). After 3 weeks of culture in Myelocult medium, the cells from the supernatant and the adherent layer (after enzymatic digestion) were seeded in methylcellulose in order to assess CFU-GM (A) and BFU-E (B) output (mean ± SD of three different bone marrow samples, each performed in triplicate). White columns: CFU-C mean ± SD obtained from 1 × 10^3 CD34$^+$ cells seeded in a 3.5 mm well under contact conditions. Gray columns: CFU-C mean ± SD obtained from 1 × 10^3 CD34$^+$ cells seeded in a 3.5 mm well under transwell conditions.

stem cell phenotype [28,29]. When confirmed and further expanded by means of phenotype and functional studies, these data may be important in defining lineage relationships between mesenchymal and hematopoietic stem cells.

The function of the LNGFR on MCs is still unknown, although it has been shown to be involved in a variety of functions, including morphogenesis [30,31], growth factor presentation [32], and, more recently, apoptosis in response to NGF stimulation [33]. LNGFR is highly expressed on directly isolated BM MCs and is maintained in unstimulated in vitro cultures, but it is rapidly down-regulated upon differentiation. When grown in the presence of bFGF at high doses, these cells rapidly differentiated into mature fibroblasts that lost CFU-F activity, osteoblastic/adipogenic differentiation capability, and LNGFR expression. The developmentally programmed loss of expression of LNGFR on MSCs is reminiscent of what was observed in the case of the hematopoietic CD34 antigen [34] and the Stro-1 antigen [8]. Therefore, LNGFR may be a marker of resting primitive MSCs and exploited to isolate these progenitors having potential applications in gene therapy and transplantation.

The potential of MSCs as vehicles for gene delivery or protein production has been indicated by many authors [6,35–37]. The availability of pure, primitive, actively cycling MSCs may improve the efficiency of a number of gene therapy protocols. Furthermore, the use of MSCs in allogeneic BM transplantation has been reported to improve engraftment, reduce alloreactivity [38], and correct congenital MC defects [39]. Transplantation of pure primitive MSCs rather than stromal cells at different maturation stages may prove to be advantageous in the clinical setting.

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